CHARACTERIZATION OF INFLUENZA A VIRUS M2 CYTOPLASMIC TAIL AMINO ACID RESIDUE POSITION 83 AND 86 MUTATIONS

by
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ABSTRACT

Influenza is a world-wide infectious disease caused by influenza virus and results in much death annually. One of the key methods contributing to the prevention of this disease is influenza vaccines, which include live attenuated influenza vaccine(LAIV). Although the sequence of LAIV strain has been revealed more than 15 years, we still do not fully understand which part of LAIV mutated genes mainly contribute to the phenotype of attenuation.

Based on prior researches, we tested the hypothesis that the amino acid(aa) residue position 83 and 86 of influenza A virus M2 cytoplasmic tail domain are responsible for virus replication. To study that, we generated panels of viruses with single amino acid mutations at target position based on A/Udorn/72 influenza A virus backbone. With verifying their sequences, plaques in MDCK cells and growth curves in both MDCK cells and primary human nasal epithelial cells are produced for identifying characteristics of different mutations. We conclude that specific mutations at position 83 and 86 of M2 protein cytoplasmic tail domain can change the kinetics and amount of progeny virus produced in A/Udorn/72 influenza A virus replication.

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Chapter 1: General Introduction

Influenza

Influenza as a disease in humans. Influenza viruses that cause significant disease in humans are classified into 3 types- A, B and C(3). Types A and B are the most significant human pathogens as they are the causal agents of seasonal influenza(4). Influenza C virus causes a mild infection. Influenza disease is characterized by varying severity of febrile and respiratory symptoms. While illness from seasonal strains generally lasts one to two weeks, influenza can cause hospitalization and death. The virus transmits through respiratory droplets and primarily infects epithelial cells of human respiratory tract(19). Populations at high risk of severe influenza disease burden including children younger than 5 years old(especially children younger than 2 years old), adults of 65 years old or elder, pregnant women, and Residents of nursing homes and other long-term care facilities(20).

Influenza as a disease in animals. Influenza is a disease common in a number animal species including horses, pigs, birds, wild aquatic mammals and domesticated animals. Type A virus is the important type as far as cross-species infections are concerned. It is distributed worldwide and usually causes a mild respiratory disease in animals. However, all species have their specific influenza A virus subtypes that circulate within them. The exception

is migratory waterfowl, which are hosts to all known subtypes of influenza A viruses.

Birds, especially aquatic birds represent a vast reservoir of type A influenza viruses. These viruses have the capacity to spread to many mammals and sometimes cause high morbidity and mortality. Avian influenza is an extremely contagious and aggressive disease that causes rapid systemic illness and death in susceptible birds. Domestic chickens and turkeys are most severely affected; mortality in these birds often exceeds 50%. Estimates of global high pathogenic avian virus loss from the outbreaks since 2003 run into billions. Although all had serious consequences for the poultry industry, most remained geographically circumscribed. From the recent findings(52), it can be assumed that interspecies transmission of influenza A viruses occurs more frequently than we think, mainly from birds to mammalian species. Although the outbreaks in poultry have weakened economies and jeopardized food security, the greatest concern for human health is the risk that present conditions could give rise to an influenza pandemic(22).

Both avian and swine influenza viruses have caused human pandemics. The transmission of avian influenza viruses or virus genes to humans is postulated to occur through pigs that act as the intermediate host. This involves either multiple mutational or gene segment reassortant events which generate viruses with recombinant genome segments that are highly pathogenic to human(21, 22, 23).

Public Health burden of influenza

There are two major types of influenza diseases: seasonal influenza and pandemic influenza. Seasonal influenza diseases cause annual epidemics that peak during winter in temperate regions and circulate worldwide. It occurs globally with an annual attack rate estimated at 5-10% in adults and 20-30% in children, which result in about 3 to 5 million cases of severe illness and about 250000 to 500000 deaths. Vaccination is the most effective way to prevent influenza illness, but its effectiveness depends on how circulating viruses are well-matched with vaccine viruses. The circulating influenza viruses are constantly changing due to antigenic drift mechanism(26).

Pandemic influenza diseases is an epidemic of an influenza virus that spreads on a worldwide scale and infects a large proportion of the human population. It can occur when a non-human(novel) influenza virus gains the ability for efficient and sustained human-to-human transmission and then spreads globally. There needs to be little pre-existing immunity in the human population for a pandemic to occur and this emergence of a new antigenic type of influenza into humans is called an “antigenic shift” mechanism. Pandemic influenza viruses cause more severe symptoms than seasonal ones, cause more total cases than seasonal influenza and have a larger impact on the general public, domestic and world economy. People have little or no

immunity to these viruses because they have no previous exposure to the virus, since pandemic viruses have proteins derived from swine or avian influenza segments.(29) There are 4 major flu pandemics recorded in human history, which happened in 1918-1919("Spanish flu", estimated 50 million people died), 1957-1958(69800 people died in US), 1968-1969(Hong Kong flu virus), and 2009-2010(H1N1 swine flu, 8870-18300 died)(27).

Influenza disease is considered a worldwide public health problem according to huge disease burden. Escalating medical costs have increased the need to quantify the burden of influenza. Influenza is often under-reported, since the illness may be confused with other viral illnesses, which may cost more to detect. In addition to the direct costs of medical care, the indirect costs of influenza are substantial and stem largely from absenteeism and loss of work productivity. Estimates of the cost of influenza in the USA, France and Germany have shown that indirect costs can be 5- to 10-fold higher than direct costs. Other costs include impaired performance, which can reduce reaction times, and adverse effects on the quality of life of patients and their families. The main approach to the control of influenza and its associated costs is the administration of vaccines. Although vaccines are widely effective, the shortcomings of present vaccines, which include manufacturing limitations that prevent guaranteed adequate supply of vaccine, the difficulty in matching vaccines to circulating strains and the need for administration by injection, highlight the need for complementary treatment(24). The

economical burden of influenza is estimated to be 87 billion dollars per year in US(25). A pandemic could result in losses of between 0.5% and 4.3% in the United Kingdom Gross Domestic Product(GDP), equals 134 to 1151 billion dollars(28).

Influenza Biology

Virus structure. Influenza viruses belong to the *Orthomyxoviridae* family of viruses. They are enveloped viruses with a segmented, negative sense RNA genome(Fig.1). The number of RNA segments compromising the viral genome varies, with influenza A and B viruses having 8 segments and influenza C virus having only 7 segments. The 8 segments of the influenza A virus genome are named after the major protein encoded by it - PB2, PB1, PA, NP, NS, HA, NA and M(6). These RNA segments encode for 11 viral proteins- two envelope glycoproteins HA and NA, three polymerase proteins PA, PB1 and PB2, two matrix proteins M1 and M2, three non-structural proteins NS1, NS2 ,PB1-F2 and nucleoprotein NP(7). Among them, PB1, PB2, PA and NP combine viral RNA genes to build viral ribonucleoprotein complexes(vRNPs). Influenza A and B viruses are spherical or filamentous in shape, with spherical forms on the order of 100nm in diameter and the filamentous forms often in excess of 300nm in length.

Influenza A virus infection and replication. The life cycle of influenza

virus starts with virus entry(Fig.2). HA protein on the surface of virus particles mediates the binding of the virus to the host cell sialic acid residues that have a 2,6 linkage to the adjacent galactose residue in humans or a 2,3 linkage in birds. Bound virus then enters the cell through 4 known mechanisms, called clathrin-mediated endocytosis(30), caveolae, macropinocytosis, and non-clathrin non-caveolae mechanism(31).

The internalized virion is then in an endosome. The low pH environment of the endosome induces conformational changes in HA that expose a fusion peptide at the N-terminus of HA2. Then the fusion peptide inserts into the endosomal membrane, pulling the viral and endosomal membranes together for fusion. Simultaneously, the M2 protein selectively pumps protons from the endosome lumen into the virion interior, which disrupts the protein interactions between the vRNP complexes and M1 protein. Free vRNP complexes then travel through the fused membrane pore and go into the cytosol.

Every viral RNA segment is bound by NP(nucleoprotein) and the polymerase proteins(PB1, PB2 and PA which form transcriptase complex). All of them form the viral ribonucleoprotein(vRNP)(32). Influenza genome replication takes place in nucleus. The vRNP, however, is too large to enter the nucleus passively, so they enter in via binding to import in molecules that mediate transport through the nuclear pore complex. The dissociation of M1 from vRNP exposes nuclear localization signals(NLS) on NP, which mediate

nuclear import(33). The PB2, PB1 and PA encode the RNA-dependent RNA polymerase(RdRp), which functions to produce both messenger RNA(mRNA) and genomic RNA. For genomic replication, virus RNA(vRNA) is first converted into positive-strand RNA by the RdRp. The RdRp also makes mRNA by using 5' ends of host mRNAs that are cleaved by the RdRp from cellular pre-mRNAs(34). A 3' poly A tail is added to the viral mRNA by polymerase stuttering(35). Unspliced and spliced mRNA transcripts leave the nucleus and are translated into proteins in the cytoplasm. All vRNPs that leave the nucleus are associated with M1 protein, which masks the NLS on the NP protein and exposes the nuclear export signals(NES) on the NS2/NEP protein. NS2/NEP mediates vRNP nuclear export. The unspliced and spliced vrial mRNAs are translated near the endoplasmic reticulum(ER) by host ribosomes, then 10 to 11 viral proteins are produced.

After translation, the membrane proteins HA, NA, M2 enter the ER where they fold and HA and NA are glycosylated(36). The proteins are then transported to the Golgi complex where HA and M2 are palmitoylated(37). The three proteins then reach the assembly site(lipid rafts) guided by apical sorting signals(38).

With membrane proteins at the plasma membrane, 8 vRNP segments are packaged. The most prevailing model for vRNP packaging is the selective incorporation model. This model suggests that on the end of each vRNP segment, there are distinct packaging signals(39) which leads to the correct

packaging of 8 distinct RNA segments.

Once the genome is completely enveloped, the viral membrane separates from the cellular membrane via membrane fission. The neuraminidase activity of NA cleaves the connection between HA protein and host cell sialic acid residues, leading to the release of complete influenza virion(40). Antiviral drugs Oseltamivir(41) and Zanamivir(42) are neuraminidase inhibitors.

Functions of the M2 protein. The M2 protein is one of the translation products of the influenza A virus M segment. It is an integral membrane protein 97 amino acids(aa) in length. M2 forms a tetramer and functions as an ion channel during virus entry. After the influenza A virus particle enters the cell through endocytosis, the M2 protein shuttles protons from the lumen of the endosome into the interior of virion. This changes the pH of the virion interior, leading to conformational changes in the M1 protein which disrupt the M1-vRNP association. vRNPs are subsequently released into the cytoplasm and can be imported into the nucleus of the cell to initiate RNA transcription and replication(2).

M2 has three major domains: i) the extracellular domain(aa position 1-24), ii) the transmembrane domain(aa position 24-44), and iii) the cytoplasmic tail domain(aa position 44-97). In particular, the cytoplasmic tail of M2 has been associated with a number of critical functions including viral membrane scission and coordination of viral genome packaging. Truncations and amino acid substitutions in the M2 cytoplasmic tail have identified several regions

that are critical for efficient production of influenza A virus particles. Amino acids at positions 82 to 89 of M2 protein are important for virus replication, but scanning alanine mutagenesis of this region failed to identify critical amino acids(15). Interestingly, positions 83 and 86 within this sequence are Ala and were not mutated in this study, suggesting that alanine residues at those positions may be important for M2 function.

Epithelial cells as a target for virus infection. The main targets of influenza virus in humans are the columnar epithelial cells of the human respiratory tract. In influenza infection, the receptor binding site of viral hemagglutinin(HA) is required for binding to galactose bound sialic acid on the surface of host cells(43). Cleavage of sialic acid is also essential for virus release from infected cells and is the functional role of viral neuraminidase(44).

In this study, we used primary differentiated human nasal epithelial cell(hNEC) cultures isolated from healthy tissue of male and female donors(45,46,47) to study the replication of viruses. Nasal epithelial cells are the primary cell type infected with IAV and these cultures allowed us to investigate IAV infection and pathogenesis in a culture system derived from that tissue.

Influenza Vaccines

The most effective means to control influenza virus infection is vaccination. Although there are various formulations, influenza vaccines currently have two main categories: inactivated influenza vaccine(IIV) and live attenuated influenza vaccines(LAIV)(11). The primary protection mediated by influenza vaccines is through antibodies that recognize the HA and NA proteins.

Although there are some new ways to produce influenza vaccine, the most common way is using an egg-based manufacturing process, which has already existed for more than 70 years. Vaccine viruses are injected into fertilized hen's eggs and incubated for several days to allow the viruses to replicate. The virus-containing fluid is harvested from the eggs. For IIV, the influenza viruses for the vaccine are then inactivated and virus antigen is partially purified. The manufacturing process continues with purification and testing. For the LAIV the viruses are weakened but not killed. The manufacturers then put doses into vials, syringes, or nasal sprayers for later testing and selling. This production method requires large numbers of chicken eggs to produce vaccine and usually takes the longest period of time to produce vaccine(49).

There are several methods of influenza vaccination. For IIV, intramuscular and intradermal routes are the common ones, which deliver the vaccine through a needle and syringe direct into human body. For LAIV, however, it is used as nasal spray. The vaccine is sprayed into the patient's nose for

inoculation through a specially designed tool(50).

To assess the immunogenicity of a vaccine, the hemagglutination assay (HA) and the hemagglutination inhibition assay (HI) are available for quantitating the relative concentration of influenza virus and anti-viral antibodies. HA and HI apply the process of hemagglutination, in which sialic acid receptors on the surface of red blood cells (RBCs) bind to the hemagglutinin glycoprotein found on the surface of influenza virus and create a network structure with interconnected RBCs and virus particles(51). This structure maintains the RBCs in a suspension, viewed as a diffuse reddish solution. The formation of a pellet at the bottom of the tube depends on the concentrations of the virus and RBCs. When the relative virus concentration is too low, the RBCs are not constrained by the network and settle down to the bottom of the well. HI assay also adds anti-viral antibodies to interfere with the virus-RBC interaction.

Inactivated influenza vaccines. Inactivated influenza vaccine, also called “flu shot”, is the major type of influenza vaccine. It contains no live viruses but killed viruses or their subunits which can trigger the immune response of human body but would not cause flu disease. Since the virus is mutating all the time, each year a new influenza vaccine is made to protect against three or four viruses that are likely to cause disease in the upcoming flu season. Even when the selected strains in the vaccine do not perfectly match the circulating seasonal viruses, the vaccine can still provide some

protection(48).

Live attenuated influenza vaccines. Current live attenuated influenza vaccines are based on a cold-adapted virus called A/Ann Arbor/6/1960(A/AA) type A master donor virus. This virus encodes the cold-adapted(*ca*), temperature-sensitive(*ts*), and attenuation(*att*) phenotypes. Besides the backbone of A/AA, LAIV strain also contains the two glycoprotein genes encoding HA and NA antigens from the virus strain that is the target of the vaccine – which could be any avian, animal or human influenza virus of pandemic potential or that is circulating in humans. The virus strains that serve as donors for the vaccine are selected by the WHO every year(12,13).

The attenuation phenotype means viruses replicate less efficient and less virulent than their corresponding wild-type(WT)(2), and M segment of influenza A virus is verified to be responsible for at least part of this phenotype(14).

Antigenic drift and shift. Influenza A viruses are characterized by the hemagglutinin(HA or H) and neuraminidase(NA or N) encoded by the virus. These two proteins are classified into serotypes and subtypes. Influenza A virus has 16 types of HA and 9 types of NA. This characteristic contributes to the mutation mechanisms of influenza A virus, which are named antigenic drift and antigenic shift. These two mechanisms are believed to explain why the influenza A virus can cause epidemics annually and pandemics at frequent intervals(1).

Antigenic drift is responsible for annual influenza epidemics. Circulating influenza A viruses are constantly mutating. Minor antigenic mutations from random mutational events to HA and NA help the virus evade the existing immune response of individuals generated from previous influenza A virus exposure(9).

Antigenic shift, although less frequent, causes much more serious consequences(9). Since Influenza A virus not only infects humans, but also infects other animals like birds and swines, , the bird influenza A virus strains can sometimes jump from aquatic birds to humans. If the cell is infected by both human influenza A strain and bird influenza A virus strain, genes from two strains mix together to yield a new strain(10). This major change of antigenic type of HA or NA, named antigenic shift, is responsible for influenza pandemics, which leads to widespread infection, societal disruption and global mortality measured in the millions(9).

We sought to test the hypothesis that influenza A virus M2 protein 83 and 86 position amino acids are important for virus attenuation. The result of this study would provide us genome target of attenuating influenza A virus, which could contribute to designing novel live attenuated influenza vaccine.

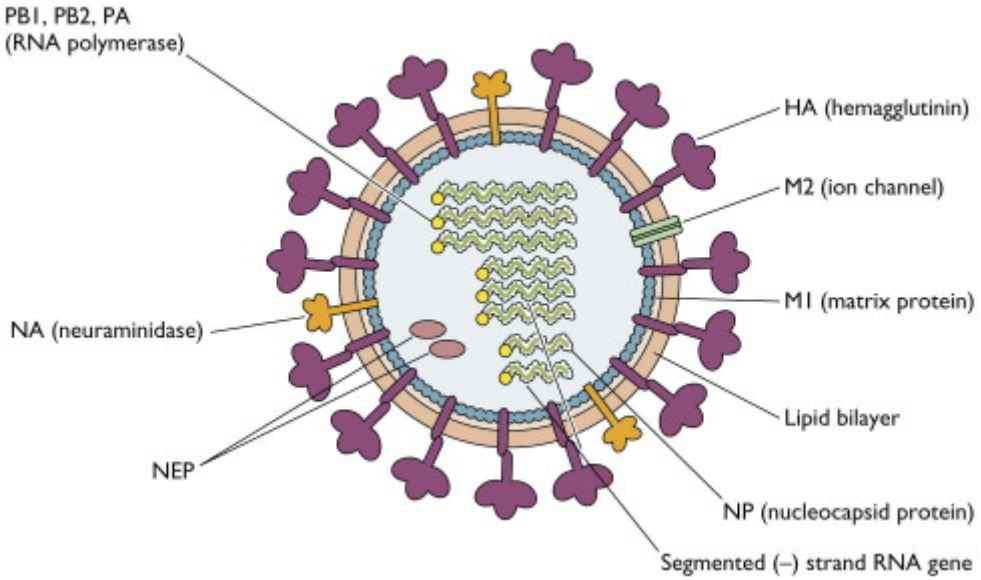


Figure. 1. Structure of Influenza A Virus(Flint, S. J. et al. Principles of Virology)

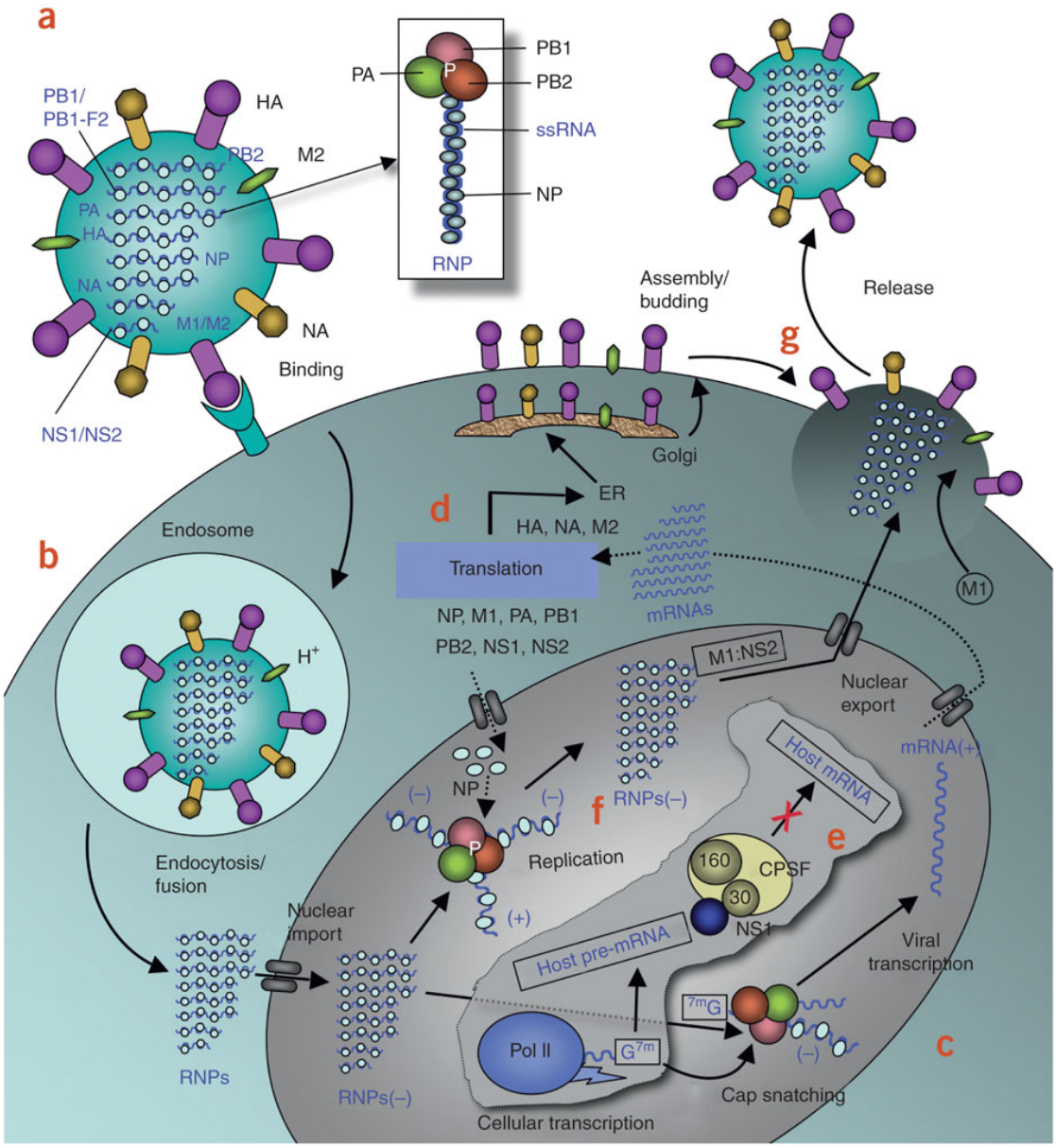


Figure. 2. Life Cycle of Influenza A Virus(Das, Kalyan, et al. "Structures of influenza A proteins and insights into antiviral drug targets." Nature structural & molecular biology 17.5(2010): 530-538.)

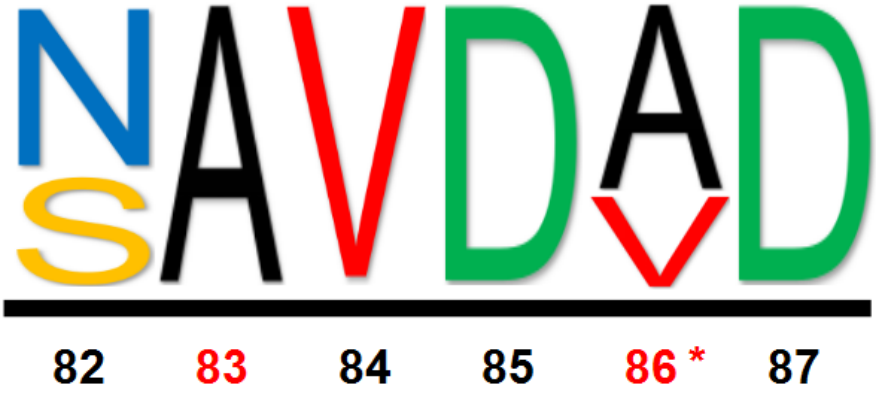


Figure. 3. Amino Acid Frequency of amino acids at positions 82-87 of the Influenza A virus M2 protein cytoplasmic tail. The relative size of the amino acid abbreviations at each position represents their relative frequencies in human influenza A virus M2 sequences present in Influenza Research Database. The asterisk(*) indicates position 86 which is encoded by S in live attenuated influenza virus(LAIV) vaccine. It has less than 0.1% relative frequency and is therefore not visible in this representation.

Chapter 2: Characterization of M2-A86 mutated viruses

BACKGROUND:

The M2 protein is one of the translation products of the M segment of Influenza A virus. It acts as an ion channel to shuttle hydrogen ions from the lumen of endosome into the interior of the virion during virus entry into cells. This disrupts the interactions between viral proteins and allows for the dissociation of the viral ribonucleoprotein(vRNP) complexes from the site of virus-endosome membrane fusion so the vRNPs can be transported to the cell nucleus to initiate viral gene expression.

The wild-type M2 protein gene sequence has 97 amino acids, and amino acids 44 to 97 are considered to be the cytoplasmic tail domain. Panels of M2 proteins encoding mutations of these amino acids have been generated and tested for their effects on virus replication. Truncating the M2 cytoplasmic tail at position 82 but not after position 90 reduced infectious virus production, suggesting a region between amino acids 82 and 90 was critical for infectious virus production. Alanine-scanning mutagenesis across this region did not, however, identify critical amino acids mediating this attenuation.

In previous analysis, the two alanines at positions 83 and 86 were not mutated so we hypothesize that these two residues may be critical for

supporting influenza A virus replication. The relative frequencies of amino acid in position 86 in all human influenza A virus show that about 2/3 of viruses have alanine at this position, while around 1/3 have valine(54). However, for LAIV virus strains, serine appears at this position. This is also the only different amino acid in the M2 protein between wild type influenza A virus A/Ann Arbor/6/60 strain and the LAIV strain derived from it [57]. The Chou & Fasman algorithm, which predicts secondary structure, indicates that region 82-90 of the M2 cytoplasmic tail forms a structure dominated by alpha-helixes. When alanine is changed to serine, the secondary structure of protein would change and disrupt the continuous alpha-helix domain(Fig.4), while alanine scanning would not do that [55,56].

In this study, we hypothesize that amino acid position 86 of influenza A virus M2 protein is important for virus replication. Recombinant viruses encoding M2 proteins with mutations at position 86 were rescued and characterized for their replication in MDCK cells and hNEC cultures. These results suggest additional amino acids may be contributing to the attenuation of LAIV.

MATERIALS AND METHODS

Plasmids. The plasmid pHH21 M-Udorn encodes the influenza A/Udorn/72 virus M segment under the control of the human RNA polymerase I promotor and murine RNA polymerase I terminator(53, 58). The pHH21 M-Udorn plasmid was altered using Quikchange Site-Directed Mutagenesis(Stratagene) protocol to introduce required A86 series mutations. The sequence of the forward and reverse mutagenesis primers used to introduce all mutations is given in Table 1. The PCR products were digested using Dpn1 enzyme to remove parental supercoiled DNA and then transformed into competent bacterial(DH5α) cells. DNA from several bacterial clones was extracted using QIAprep Spin Miniprep Kit(Qiagen). The DNA was sequenced for the appropriate mutations by sequencing the with M segment-specific primers(named FluA1 and FluA2 - given in Table 1). This sequencing was done at the Synthesis & Sequencing Facility of the Johns Hopkins University(Baltimore, MD) using Applied Biosystems 3730xl DNA Analyzer and dye terminator sequencing technology. The concentration of plasmid DNA was determined using the NanoDrop spectrophotometer ND-1000(Thermo Fisher Scientific).

Cell lines. Madin Darby canine kidney(MDCK) cells were maintained in DMEM with 10% fetal bovine serum(FBS), 100U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-Glutamine at 37°C with 5% CO_2 and were passaged

1:10 every 3 days.

293Ts were maintained in DMEM with 10% FBS, 100U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-Glutamine at 37°C with 5% CO_2 and were passaged 1:10 every 3 days. 6-well plates coated with poly-L-lysine were seeded with approximately 1.2*10^6 cells per well 18-24 hours prior to transfection to target 60-70% confluence at the time of transfection.

Human nasal epithelial cell cultures. Human nasal epithelial cells(hNEC) were collected from non-diseased nasal mucosa of patients undergoing endoscopic sinonasal surgery for non-sinusitis indications, including dacryocystorhinostomy, approach to anterior skull base pathology, or removal of benign nasal masses.(Fischer et al) The cells were collected from 2 male and 2 female donors. The cells were differentiated at an air-liquid interface(ALI) in 24-well Falcon filter inserts(0.4-µM pore; 0.33cm^2; Becton Dickinson) before infection, using ALI medium as basolateral medium.

Recombinant viruses. The 12 plasmid based recombinant virus rescue system(58, 60) was used to generate all the mutated viruses. 293T cells were transfected with 0.5µg of pHH21 plasmids encoding A/Udorn/72 PB1, PB2, PA, HA, NA, NP, NS and M; and plasmids(under the control of cellular RNA polymerase II promoters) encoding A/Udorn/72 PB1, PB2, NP(1µg each) and PA(0.2µg). Transfection reagent TransIT-LT-1(LT1)(Mirus, Madison, WI) was mixed with OptiMEM medium(Gibco, Carlsbad, CA) and incubated at room temperature for 15minutes at a ratio of 2µl LT1 to 1µg plasmid DNA. The

plasmids were mixed(each transfection well with appropriate M segment) with indicated quantities and incubated with LT1-OptiMEM mixture for another 15 minutes. Medium was removed from 293T cells in 6-well plates and replaced with 2ml of OptiMEM. The corresponding transfection solution was added to each well. The plates were incubated at 32°C with 5% CO_2. After 24 hours, N-acetyl trypsin(NAT)(Sigma, St.Louis, MO) was added to a final concentration of 10μg/ml to each well.. After incubation for 4 hours at 32°C with 5% CO_2, approximately 5^10^5 MDCK cells in 100μl OptiMEM were added to each well of 293T cells. The plates were incubated at 37°C with 5% CO_2. 1ml of transfected cell supernatant were collected and replaced with 1ml DMEM with 4μg/ml NAT, 100U/ml Penicillin 100μg/ml Streptomycin, 2mM L-Glutamine and 0.5% bovine serum albumin(BSA)(Sigma)(Infectious medium with NAT, IM+NAT) daily until obvious signs of cytopathic effect were visible.

The recombinant viruses used in this study were all generated in the A/Udorn/72(rUdorn) genetic background(60). The viruses with mutations at position 86 are listed in Table 2 and include mutation to amino acids V, S, M, E and K.

$TCID_{50}$ assay Fifty percent tissue culture infectious dose($TCID_{50}$) was determined in 96-well plates of MDCK cells using a ten-fold dilution series of each virus sample in IM+NAT. 120μl of each dilution was added to each of 6 wells and the plates were incubated at 32°C with 5% CO_2 for 7 days. The cells were then fixed with 4% formaldehyde at least 1 hour and stained with

napthol blue black for at least 4 hours. The cytopathic effect was scored visually and the $TCID_{50}$ value for the virus sample was obtained using the Reed and Muench calculation.

Plaque assay and plaque picking. Plaque assays were done to purify virus clones from transfected cell-supernatants and to quantify the plaque morphology of virus strains. 6-well plates were seeded with MDCK cells and allowed to reach 90-100% confluence. 100µl of transfected-cell supernatant or virus-infected cell supernatant was serially diluted ten-fold with IM+NAT. Virus dilutions were added to each well and incubated at room temperature for one hour with rocking gently. Then the wells were covered with 2% agarose mixed with 2XMEM with 4µg/ml NAT. Plates were incubated at 32°C with 5% CO_2 for 72-96 hours. Plaques were picked using a 1ml pipette, then placed in tubes containing IM and stored at -70°C . The plates were fixed with 4% formaldehyde in PBS for 1 hour and stained with napthol blue black for at least 4 hours to reveal the plaques.

To quantify plaque area, images of the plaques were taken using a dissecting microscope with a Olympus DP-70 color camera and including a standard ruler in the image. Photos opened by software ImageJ were turned into 8-bit images photo background was subtracted with “Rolling ball radius 50.0 pixels” and ticking “Light background” and “Sliding paraboloid” items. Image threshold was adjusted to make the plaques easy to recognize. Using “Paintbrush tool”, the plaque borders were highlighted manually, then filled to

create a black and white image. Plaque area data from ImageJ with Prism 6.

Virus infection for generating virus stocks Virus infections were done using MDCK cells that were approximately 100% confluent in T150(150cm^2) flasks. 100µl of purified virus(plaque picking products) was added to 5ml of IM+NAT and this was incubated in the T150 flasks for 1hour at room temperature with gently rocking. 15ml more IM+NAT was added and flasks were incubated at 32°C. The cells were monitored daily for cytopathic effect(CPE) and the supernatants were harvested when approximately 40-50% of cells had detached from the flask bottom surface. The harvested samples are seed stocks. The seed stocks were sequenced for specific mutations and infectious virus titrated by $TCID_{50}$ assay. When the seed stock sequence was verified, working stocks(at MOI=0.001) were generated from seed stocks in the same way as above. The working stocks were also titrated by TCID50, sequenced and used in all experiments.

Low MOI growth curves(GCs). To determine virus replication kinetics, different panels of mutated A86 series viruses were used in 24-well plates with near 100% confluence MDCK cells or fully differentiated hNECs. Viruses were diluted to a low multiplicity of infection(MOI) of 0.001 infectious virus particles per cell in IM+NAT(MDCK) or IM(hNEC). The virus inoculum was then added to wells and incubated for 1 hour with rocking at room temperature(MDCK GCs) or under 32°C or 37°C with 5% CO_2 for 1 hour(hNEC GCs). For MDCK infections, the inoculum was then aspirated and replaced with 500µl IM+NAT,

and samples were taken at 1, 12, 24, 36, 48, and later every 24hours and replaced with fresh 500µl IM+NAT. For hNEC infections, The inoculum was then aspirated and at indicated times post infection(1, 12, 24, 36, 48, 72, 96, 120, 144 hours post infection, hpi), 100µl IM was added to each well, the plates incubated at 32°C or 37°C with 5% CO_2 for 10 minutes, and the supernatant harvested. The basolateral medium was replaced every 48 hours.

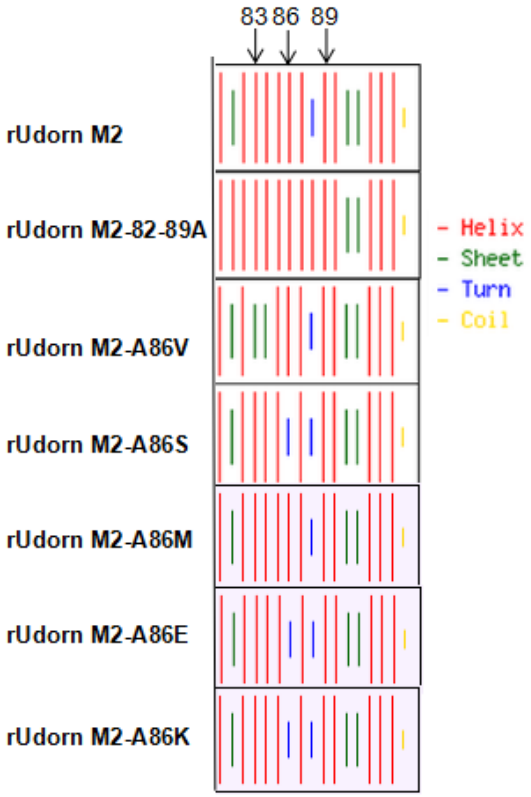


Figure. 4. Predicted secondary structure of amino acids 82-89 of the M2 protein of A/Udorn/72 and the effect of amino acid substitutions at position 86.

Plasmid	Forward Primer	Reverse Primer
pHH21 Ud-M2-A86V	5’ CAA AAT GAC TGT CGT CAA CAT CCA CAG C 3’	5’ GAA CAG CAG AGT GCT GTG GAT GTT GAC G 3’
pHH21 Ud-M2A86S	5’ CAA AAT GAC TGT CGT CAG AAT CCA CAG C 3’	5’ GAA CAG CAG AAT GCT GTG GAT TCT GAC G 3’
pHH21 Ud-M2A86M	5’ CAA AAT GAC TGT CGT CCA TAT CCA CAG C 3’	5’ GAA CAG CAG AGT GCT GTG GAT ATG GAC G 3’
pHH21 Ud-M2A86E	5’ CAA AAT GAC TGT CGT CTT CAT CCA CAG CA 3’	5’ GAA CAG CAG AGT GCT GTG GAT GAA GAC G 3’
pHH21 Ud-M2A86K	5’ CAA AAT GAC TGT CGT CCT TAT CCA CAG CA 3’	5’ GAA CAG CAG AGT GCT GTG GAT AAG GAC G 3’
pHH21 Ud-M2A83V	5' GAA CAG CAG AGT GTT GTG GAT GCT GAC G 3'	5' C GTC AGC ATC CAC AAC ACT CTG CTG TTC 3'
pHH21 Ud-M2A83P	5' GAA CAG CAG AGT CCT GTG GAT GCT GAC G 3'	5' C GTC AGC ATC CAC AGG ACT CTG CTG TTC 3'
pHH21 Ud-M2A83M	5' GAA CAG CAG AGT ATG GTG GAT GCT GAC G 3'	5' C GTC AGC ATC CAC CAT ACT CTG CTG TTC 3'
pHH21 Ud-M2A83E	5' GAA CAG CAG AGT GAA GTG GAT GCT GAC G 3'	5' C GTC AGC ATC CAC TTC ACT CTG CTG TTC 3'
pHH21 Ud-M2A83K	5' GAA CAG CAG AGT AAG GTG GAT GCT GAC G 3'	5' C GTC AGC ATC CAC CTT ACT CTG CTG TTC 3'
pHH21 Ud-M	FluA1 5’ ATA CGT CTC GTA TTA GTA GAA ACA AGG TAG 3’	FluA2 5’ CGA CGT CTC CGG GAG CAA AAG CAG GTA G 3’

Table 1. Primer sequences used in project

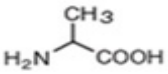
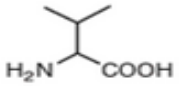
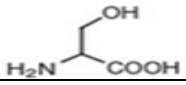
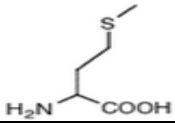
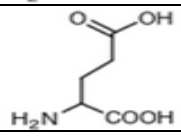
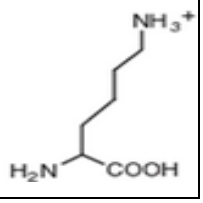
Mutation	Amino Acid(AA)	% Relative Freq	Residue Properties	AA structure
WT	Alanine	65	Small Hydrophobic	
A86V	Valine	34	Small Hydrophobic	
A86S	Serine*	0.01	Small Nucleophilic	
A86M	Methionine	0	Large Hydrophobic	
A86E	Glutamic Acid	0	Acidic	
A86K	Lysine	0	Basic	

Table 2. The viruses designed with mutations at position 86

*A86S is the LAIV strain mutation

RESULTS

Rescue of recombinant influenza viruses encoding M2-A86 mutations. Recombinant viruses expressing the panel of M2-A86 mutations shown in Table 2 were successfully rescued. The entire coding region of the M-segment from each virus was sequenced to confirm the presence of the desired mutation and to verify that no other amino acid changes were present.

Plaque assay of A/Udorn/72 viruses containing M2-A86 mutations. As a first measure to determine if mutations at M2-A86 could alter influenza A virus replication, we determined the plaque morphology and size on MDCK cells. All of the viruses are able to form discernible plaques(Fig. 5A).The area of individual plaques was then calculated and viruses containing the M2-A86S mutations generated slightly smaller plaques compared to WT viruses. This result indicates that the serine mutation, which is only present in the LAIV virus strain, negatively impacts influenza A virus plaque formation, though the effect is rather small.

Replication of recombinant viruses encoding M2-A86 mutations at 32°C. To further characterize the replication of recombinant influenza A viruses encoding M2-A86 mutations, a multiple-step growth curve was performed on MDCK and differentiated human nasal epithelial cell(hNEC) cultures. To mimic the natural infection temperature of the upper respiratory tract, the growth curves were performed at 32°C. All the recombinant viruses

reached peak virus titers at 72 hpi, but viruses encoding M2-A86V, A86M and A86K replicated with slightly faster similar kinetics at early times post infection in MDCK cells(Fig.6A).

In hNEC cultures, recombinant viruses encoding M2-A86S, A86E and A86K showed reduced kinetics of virus production and reached peak virus titer 24 hours later than the other recombinant viruses(Fig.7A). The difference between WT and M2-A86S or M2-A86E was approximately 10 fold at multiple timepoints, while the recombinant virus encoding M2 A86K showed reduced virus titers at only one time post infections, suggesting a smaller effect of this mutation on virus replication in hNEC cultures.

These data demonstrates that at 32° C in MDCK cells, M2-A86 mutations do not adversely affect virus replication and some mutations may in fact increase virus replication slightly. However, in a physiologically relevant cell culture system like hNEC cultures, the M2-A86S and M2-A86E mutations consistently reduce virus replication. To further characterize virus replication, we chose a subset of recombinant viruses – M2-A86V, S and E – to represent viruses that have slightly enhanced replication in MDCK cells or reduced replication in hNEC cultures(Fig.6B & Fig.7B).

Replication of recombinant viruses encoding M2-A86 mutations at 37°C. While the upper respiratory tract temperature is approximately 32° C, core human body temperature and therefore the temperature of the lower respiratory tract, is 37° C. The M2-A86S mutation attenuated influenza virus

replication in hNEC cultures but not in MDCK cells, a phenotype that is consistent with that observed with LAIV replication in those same cell culture systems(Fisher, et al 2015). To determine if the virus replication patterns of the M2-A86V, S and E viruses were affected by increased temperature, multi-step virus growth curves were performed at 37° C using both MDCK and hNEC cultures. At 37° C, all four viruses replicated with identical kinetics and to similar peak titers, indicating that the slightly enhanced replication of the virus encoding M2-A86V was not present at higher temperature(Fig.8). In hNECs, all three M2 mutations resulted in reduced virus replication kinetics and peak titer(Fig.9). Recombinant viruses encoding M2-A86S and M2-A86E had peak titers approximately 100-fold lower than WT virus, which are much larger than the difference less than 10-fold under 32° C. M2-A86V virus replicates better than M2-A86S and M2-A86E, but still averagely 10-fold lower than WT virus.

Taken together, this data demonstrate that the M2-A86S and M2-A86E mutations can attenuate A/Udorn/72 virus replication by reducing infectious virus production in hNEC cultures but not in MDCK cells. This attenuation is even greater at higher temperatures.

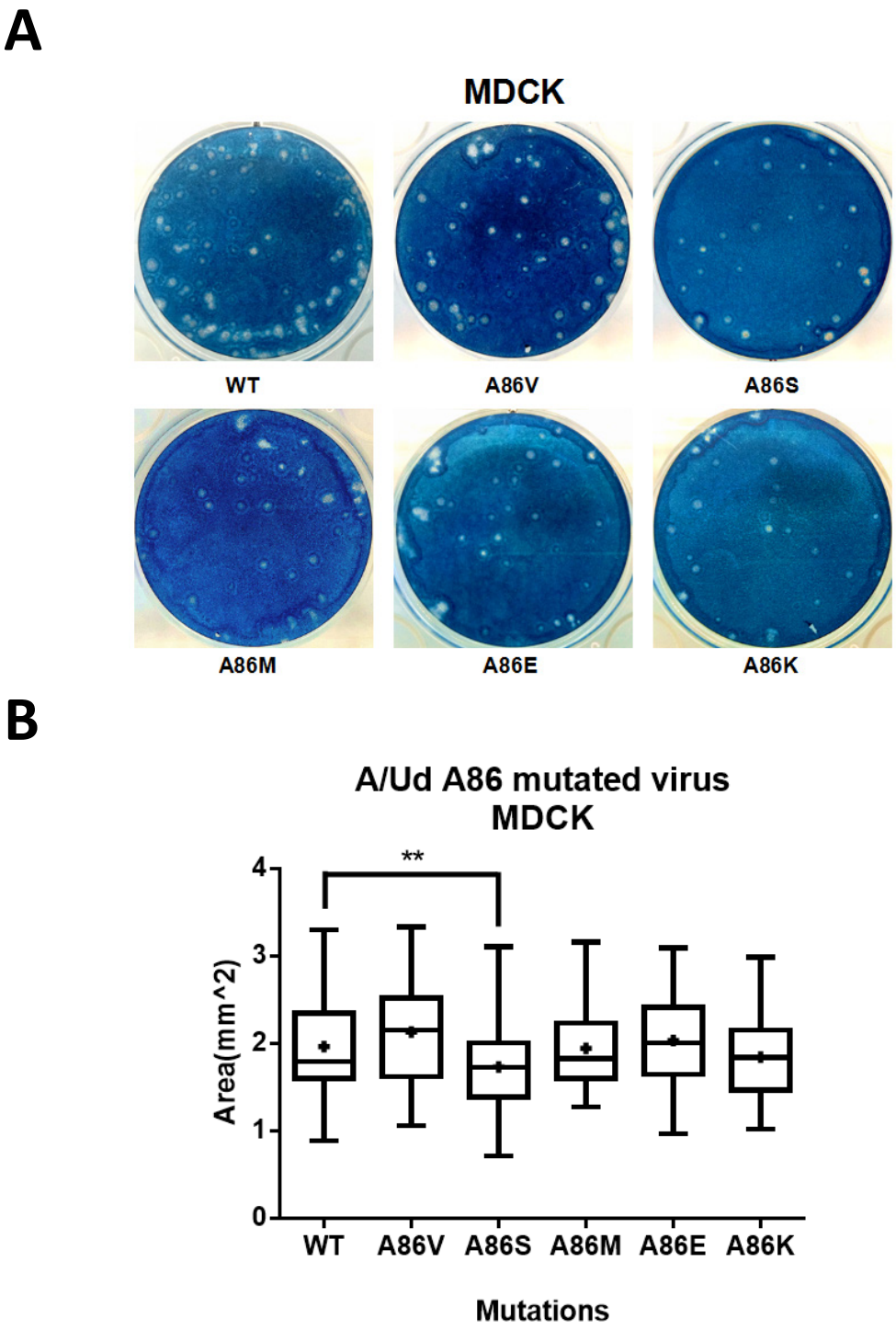
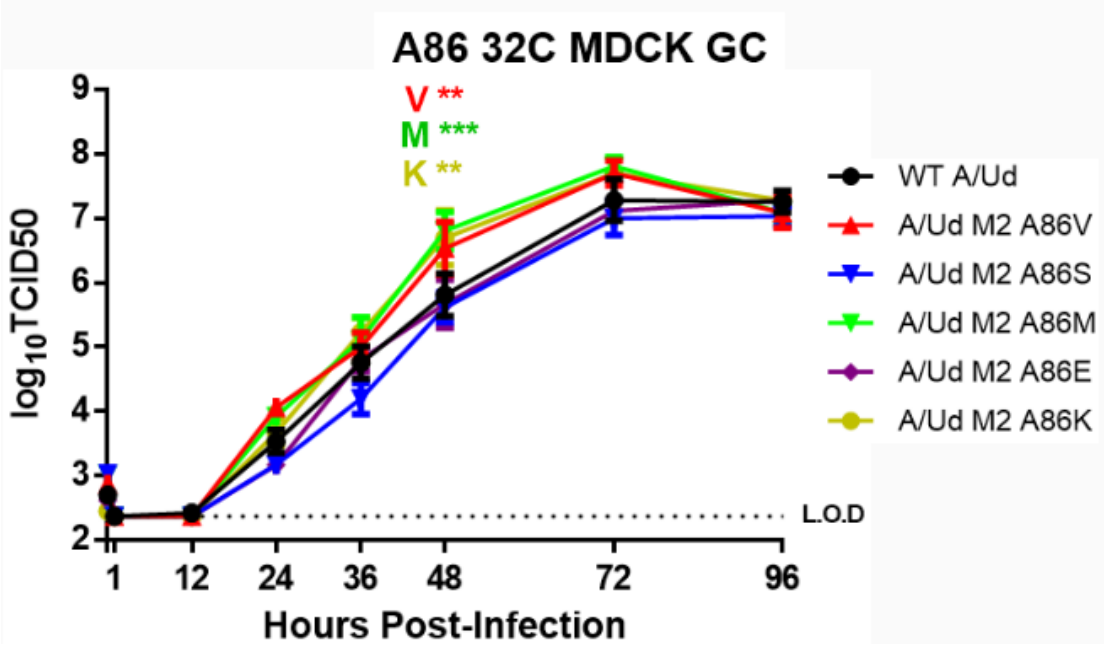


Figure. 5. Effect of amino acid substitutions at position 86 of the M2 protein on influenza A virus plaque morphology in MDCK cells.(A) Plaque morphology of recombinant influenza A viruses encoding mutations at position 86 of the M2 protein.(B) Plaque area was calculated and mean(cross), median(line), 50% percentiles(box) and range(whiskers) are shown. Significant differences were determined by student t-test: ** P≤0.01. At least 60 plaques were quantified for each virus.

A



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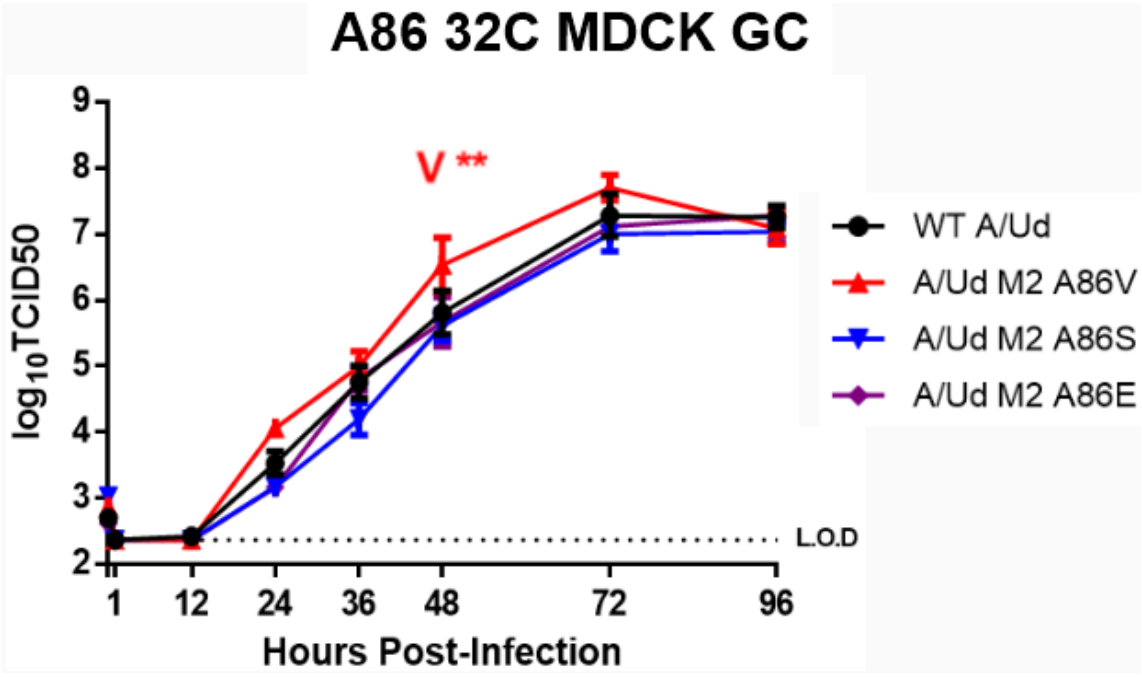
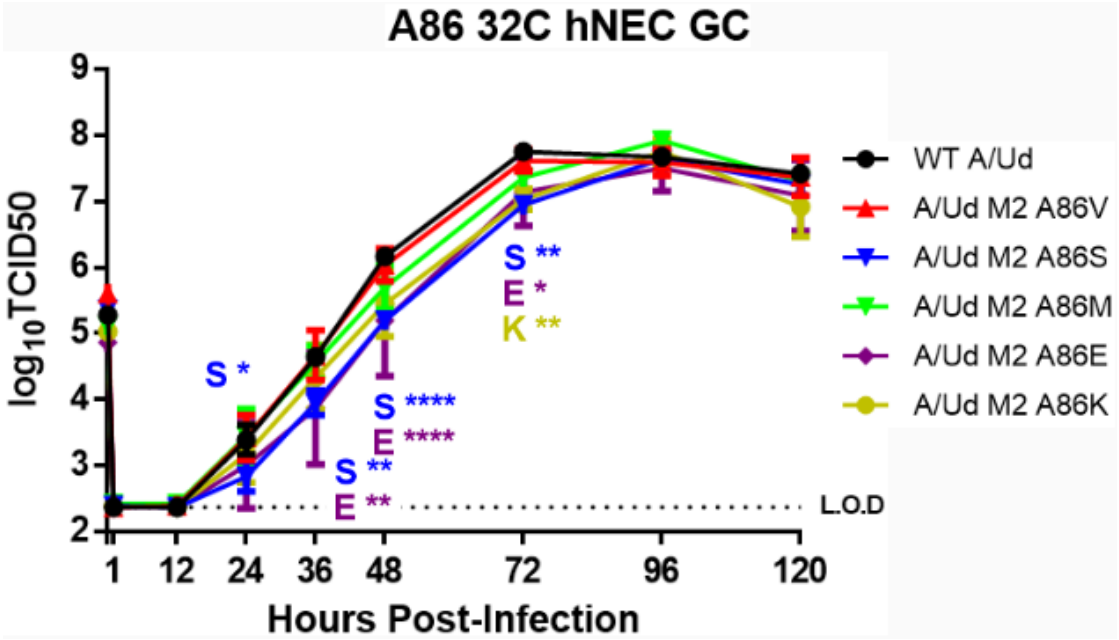


Figure. 6. Effect of amino acid substitutions at position 86 of the M2 protein on influenza A virus replication in MDCK cells at 32°C(A) A multi-step growth curve was performed on MDCK cells with the indicated recombinant viruses. In(B), representative viruses from(A) are shown. Statistical differences determined by MANOVA followed by Bonferroni post test: **=P<0.01; ***=P<0.001. L.O.D=limit of detection=2.37.

A



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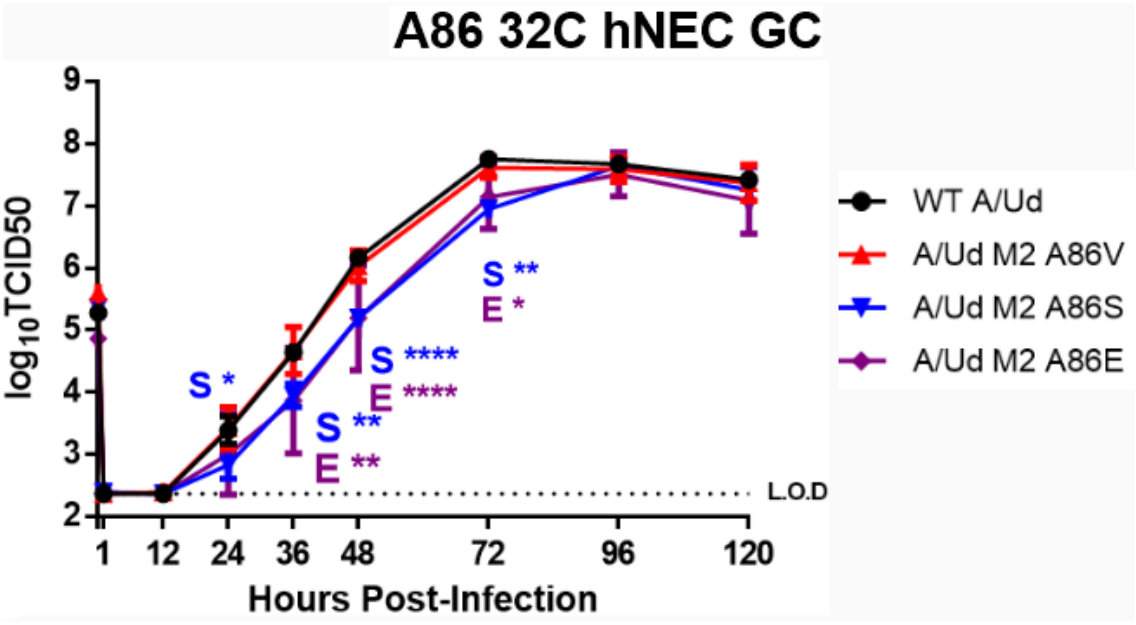


Figure. 7. Effect of amino acid substitutions at position 86 of the M2 protein on influenza A virus replication in hNEC cultures at 32°C.(A) A multistep growth curve was performed on hNEC cultures with the indicated recombinant viruses. In(B), representative viruses from(A) are shown. Statistical differences determined by MANOVA followed by Bonferroni post test: *=P<0.05; **=P<0.01; ****=P<0.0001. L.O.D=limit of detection=2.37.

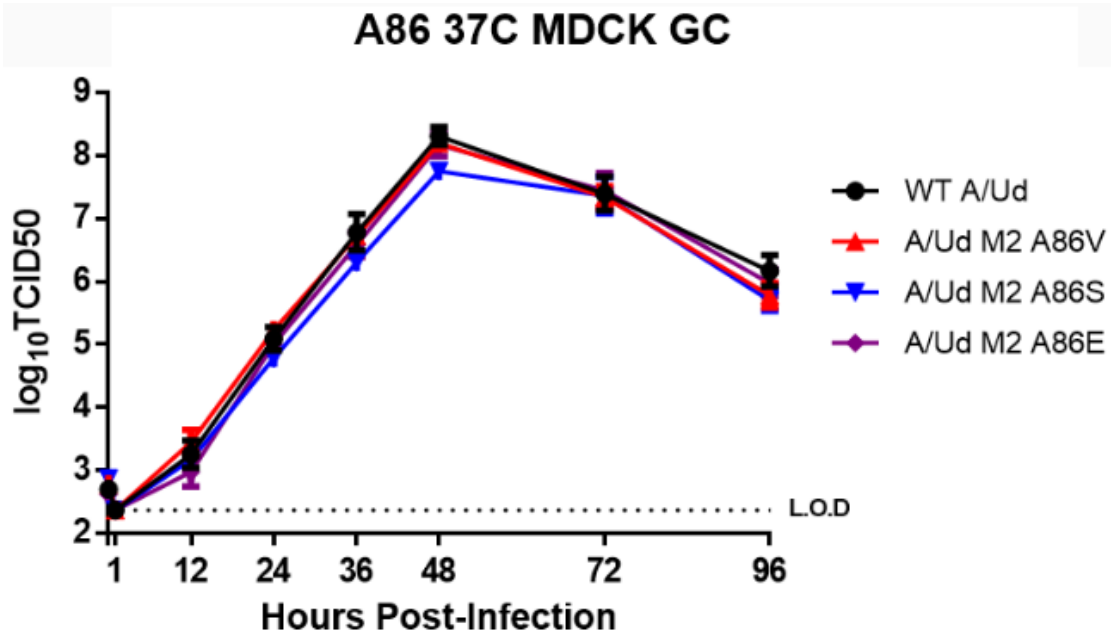


Figure. 8. Effect of amino acid substitutions A86V, A86S and A86E of the M2 protein on influenza A virus replication in MDCK cells at 37°C. A multistep growth curve was performed on MDCK cells with the indicated recombinant viruses. Statistical differences determined by MANOVA followed by Bonferroni post test. L.O.D=limit of detection=2.37.

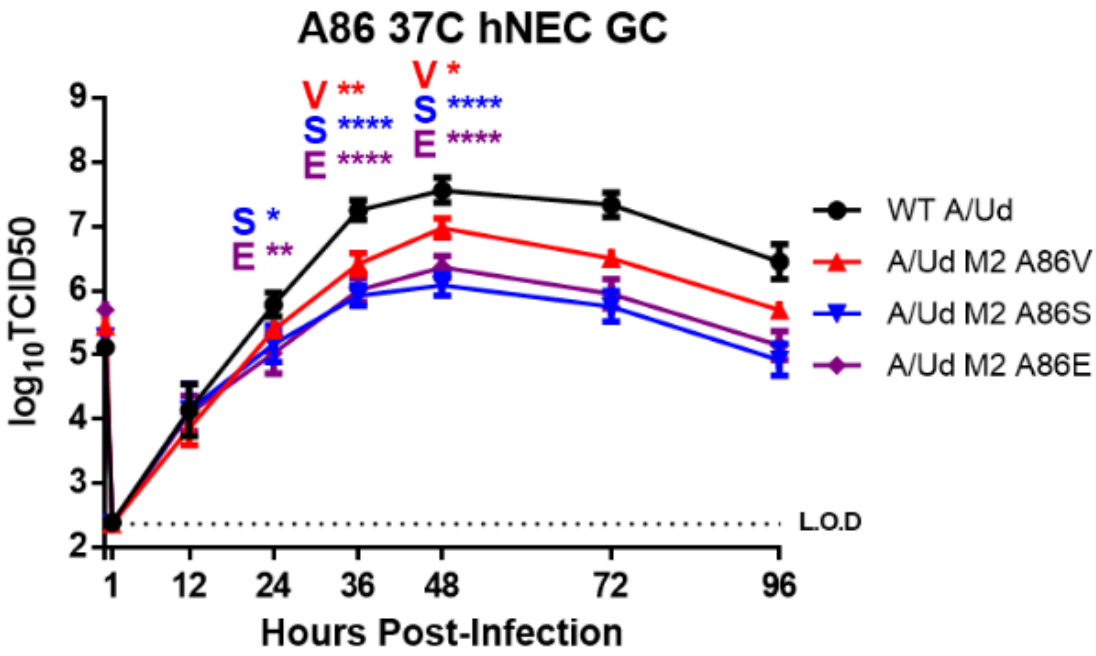


Figure. 9.Effect of amino acid substitutions A86V, A86S and A86E of the M2 protein on influenza A virus replication in hNEC cultures at 37°C.. A multistep growth curve was performed on hNEC cultures with the indicated recombinant viruses. Statistical differences determined by MANOVA followed by Bonferroni post test: **=P<0.01; ***=P<0.001. L.O.D=limit of detection=2.37.

Chapter 3: Characterization of M2-A83 mutated viruses

BACKGROUND

The M2 protein is one of the translation products of the M segment of Influenza A virus. It acts as an ion channel to shuttle hydrogen ions from the lumen of endosome into the interior of the virion during virus entry into cells. This disrupts the interactions between viral proteins and allows for the dissociation of the viral ribonucleoprotein(vRNP) complexes from the site of virus-endosome membrane fusion so the vRNPs can be transported to the cell nucleus to initiate viral gene expression.

The wild-type M2 protein gene sequence has 97 amino acids, and amino acids 44 to 97 are considered to be the cytoplasmic tail domain. Panels of M2 proteins encoding mutations of these amino acids have been generated and tested for their effects on virus replication. Truncating the M2 cytoplasmic tail at position 82 but not after position 90 reduced infectious virus production, suggesting a region between amino acids 82 and 90 was critical for infectious virus production. Alanine-scanning mutagenesis across this region did not, however, identify critical amino acids mediating this attenuation.

In previous analysis, the two alanines at positions 83 and 86 were not mutated so we hypothesize that these two residues may be critical for

supporting influenza A virus replication. The relative frequencies of amino acid in position 83 in all human influenza A virus show that more than 99% of viruses have Alanine at this 83 position(54). The Chou & Fasman algorithm, which predicts secondary structure, indicates that region 82-90 of the M2 cytoplasmic tail forms a structure dominated by alpha-helixes. When alanine is changed to proline, the secondary structure of protein would change and disrupt the continuous alpha-helix domain(Fig.10), while alanine scanning would not do that [55,56].

In this study, we hypothesize that amino acid position 83 of influenza A virus M2 protein is important for virus replication. Recombinant viruses encoding M2 proteins with mutations at position 83 were rescued and characterized for their replication in MDCK cells and hNEC cultures. These results suggest additional amino acids may be contributing to the attenuation of LAIV.

MATERIALS AND METHODS

Plasmids. The plasmid pHH21 M-Udorn encodes the influenza A/Udorn/72 virus M segment under the control of the human RNA polymerase I promotor and murine RNA polymerase I terminator(53, 58). The pHH21 M-Udorn plasmid was altered using Quikchange Site-Directed Mutagenesis(Stratagene) protocol to introduce required A83 series mutations. The sequence of the forward and reverse mutagenesis primers used to introduce all mutations is given in Table 1. The PCR products were digested using Dpn1 enzyme to remove parental supercoiled DNA and then transformed into competent bacterial(DH5α) cells. DNA from several bacterial clones was extracted using QIAprep Spin Miniprep Kit(Qiagen). The DNA was sequenced for the appropriate mutations by sequencing the with M segment-specific primers(named FluA1 and FluA2 - given in Table 1). This sequencing was done at the Synthesis & Sequencing Facility of the Johns Hopkins University(Baltimore, MD) using Applied Biosystems 3730xl DNA Analyzer and dye terminator sequencing technology. The concentration of plasmid DNA was determined using the NanoDrop spectrophotometer ND-1000(Thermo Fisher Scientific).

Cell lines. Madin Darby canine kidney(MDCK) cells were maintained in DMEM with 10% fetal bovine serum(FBS), 100U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-Glutamine at 37°C with 5% CO_2 and were passaged

1:10 every 3 days.

293Ts were maintained in DMEM with 10% FBS, 100U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-Glutamine at 37°C with 5% CO_2 and were passaged 1:10 every 3 days. 6-well plates coated with poly-L-lysine were seeded with approximately 1.2*10^6 cells per well 18-24 hours prior to transfection to target 60-70% confluence at the time of transfection.

Human nasal epithelial cell cultures. Human nasal epithelial cells(hNEC) were collected from non-diseased nasal mucosa of patients undergoing endoscopic sinonasal surgery for non-sinusitis indications, including dacryocystorhinostomy, approach to anterior skull abse pathology, or removal of benign nasal masses.(Fischer et al) The cells were collected from 1 male and 1 female donors. The cells were differentiated at an air-liquid interface(ALI) in 24-well Falcon filter inserts(0.4-µM pore; 0.33cm^2; Becton Dickinson) before infection, using ALI medium as basolateral medium.

Recombinant viruses. The 12 plasmid based recombinant virus rescue system(58, 60) was used to generate all the mutated viruses. 293T cells were transfected with 0.5µg of pHH21 plasmids encoding A/Udorn/72 PB1, PB2, PA, HA, NA, NP, NS and M; and plasmids(under the control of cellular RNA polymerase II promoters) encoding A/Udorn/72 PB1, PB2, NP(1µg each) and PA(0.2µg). Transfection reagent TransIT-LT-1(LT1)(Mirus, Madison, WI) was mixed with OptiMEM medium(Gibco, Carlsbad, CA) and incubated at room temperature for 15minutes at a ratio of 2µl LT1 to 1µg plasmid DNA. The

plasmids were mixed(each transfection well with appropriate M segment) with indicated quantities and incubated with LT1-OptiMEM mixture for another 15 minutes. Medium was removed from 293T cells in 6-well plates and replaced with 2ml of OptiMEM. The corresponding transfection solution was added to each well. The plates were incubated at 32°C with 5% CO_2. After 24 hours, N-acetyl trypsin(NAT)(Sigma, St.Louis, MO) was added to a final concentration of 10μg/ml to each well. After incubation for 4 hours at 32°C with 5% CO_2, approximately $5^{\wedge}10^5$ MDCK cells in 100μl OptiMEM were added to each well of 293T cells. The plates were incubated at 37°C with 5% CO_2. 1ml of transfected-cell supernatant were collected and replaced with 1ml DMEM with 4μg/ml NAT, 100U/ml Penicillin 100μg/ml Streptomycin, 2mM L-Glutamine and 0.5% bovine serum albumin(BSA)(Sigma)(Infectious medium with NAT, IM+NAT) daily until obvious signs of cytopathic effect were visible.

The recombinant viruses used in this study were all generated in the A/Udorn/72(rUdorn) genetic background(60). The viruses with mutations at position 83 are listed in Table 3 and include mutation to amino acids V, P, M, E and K.

$TCID_{50}$ assay Fifty percent tissue culture infectious dose($TCID_{50}$) was determined in 96-well plates of MDCK cells using a ten-fold dilution series of each virus sample in IM+NAT. 120μl of each dilution was added to each of 6 wells and the plates were incubated at 32°C with 5% CO_2 for 7 days. The cells were then fixed with 4% formaldehyde at least 1 hour and stained with

napthol blue black for at least 4 hours. The cytopathic effect was scored visually and the $TCID_{50}$ value for the virus sample was obtained using the Reed and Muench calculation.

Plaque assay and plaque picking. Plaque assays were done to purify virus clones from transfected cell-supernatants and to quantify the plaque morphology of virus strains. 6-well plates were seeded with MDCK cells and allowed to reach 90-100% confluence. 100µl of transfected-cell supernatant or virus-infected cell supernatant was serially diluted ten-fold with IM+NAT. Virus dilutions were added to each well and incubated at room temperature for one hour with rocking gently. Then the wells were covered with 2% agarose mixed with 2XMEM with 4µg/ml NAT. Plates were incubated at 32°C with 5% CO_2 for 72-96 hours. Plaques were picked using a 1ml pipette, then placed in tubes containing IM and stored at -70°C. The plates were fixed with 4% formaldehyde in PBS for 1 hour and stained with napthol blue black for at least 4 hours to reveal the plaques.

To quantify plaque area, images of the plaques were taken using a dissecting microscope with a Olympus DP-70 color camera and including a standard ruler in the image. Photos opened by software ImageJ were turned into 8-bit images photo background was subtracted with “Rolling ball radius 50.0 pixels” and ticking “Light background” and “Sliding paraboloid” items. Image threshold was adjusted to make the plaques easy to recognize. Using “Paintbrush tool”, the plaque borders were highlighted manually, then filled to

create a black and white image. Plaque area data from ImageJ was analyzed with Prism 6.

Virus infection for generating virus stocks Virus infections were done using MDCK cells that were approximately 100% confluent in T150(150cm^2) flasks. 100µl of purified virus(plaque picking products) was added to 5ml of IM+NAT and this was incubated in the T150 flasks for 1hour at room temperature with gently rocking. 15ml more IM+NAT was added and flasks were incubated at 32°C. The cells were monitored daily for cytopathic effect(CPE) and the supernatants were harvested when approximately 40-50% of cells had detached from the flask bottom surface. The harvested samples are seed stocks. The seed stocks were sequenced for specific mutations and infectious virus titrated by $TCID_{50}$ assay. When the seed stock sequence was verified, working stocks(at MOI=0.001) were generated from seed stocks in the same way as above. The working stocks were also titrated by TCID50, sequenced and used in all experiments.

Low MOI growth curves(GCs). To determine virus replication kinetics, different panels of mutated A83 series viruses were used in 24-well plates with near 100% confluence MDCK cells or fully differentiated hNECs. Viruses were diluted to a low multiplicity of infection(MOI) of 0.001 infectious virus particles per cell in IM+NAT(MDCK) or IM(hNEC). The virus inoculum was then added to wells and incubated for 1 hour with rocking at room temperature(MDCK GCs) or under 32°C with 5% CO_2 for 1 hour(hNEC GCs). For MDCK infections,

the inoculum was then aspirated and replaced with 500µl IM+NAT, and samples were taken at 1, 12, 24, 36, 48, and later every 24hours and replaced with fresh 500µl IM+NAT. For hNEC infections, The inoculum was then aspirated and at indicated times post infection(1, 12, 24, 36, 48, 72, 96, 120, 144 hours post infection, hpi), 100µl IM was added to each well, the plates incubated at 32°C with 5% CO_2 for 10 minutes, and the supernatant harvested. The basolateral medium was replaced every 48 hours.

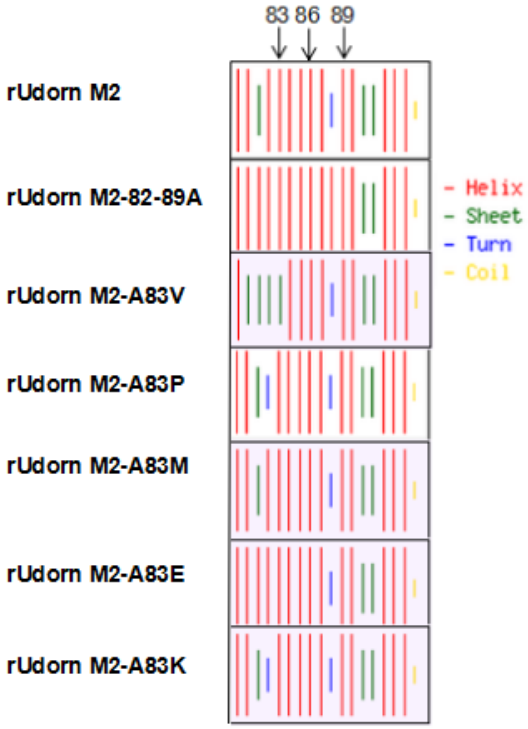


Figure. 10. Predicted secondary structure of amino acids 82-89 of the M2 protein of A/Udorn/72 and the effect of amino acid substitutions at position 83.

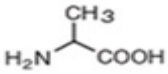
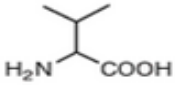
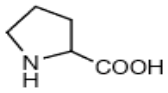
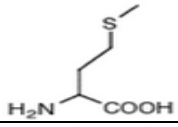
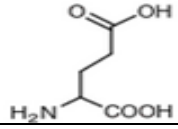
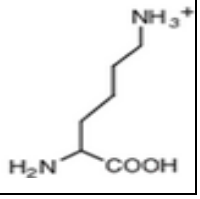
Mutation	Amino Acid(AA)	% Relative Freq	Residue Properties	AA structure
WT	Alanine	99	Small Hydrophobic	
A83V	Valine	0	Small Hydrophobic	
A83P	Proline	0	Large Hydrophobic (α-helix disruptor)	
A83M	Methionine	0	Large Hydrophobic	
A83E	Glutamic Acid	0	Acidic	
A83K	Lysine	0	Basic	

Table 3. The viruses designed with mutations at position 83

RESULTS:

Rescue of recombinant influenza viruses encoding M2-A83 mutations. Recombinant viruses expressing the panel of M2-A86 mutations shown in Table 2 were successfully rescued. The entire coding region of the M-segment from each virus was sequenced to confirm the presence of the desired mutation and to verify that no other amino acid changes were present.

Plaque assay of A/Udorn/72 viruses containing M2-A83 mutations. As a first measure to determine if mutations at M2-A83 could alter influenza A virus replication, we determined the plaque morphology and size on MDCK cells. All of the viruses are able to form discernible plaques(Fig. 11A).The area of individual plaques was then calculated and viruses containing the M2-A83E and M2-A83K mutations generated slightly larger plaques compared to WT virus. This result indicates that the glutamine and lysine mutations, neither of which appears in natural isolates of influenza A virus, enhance influenza A virus plaque formation, though the effect is rather small.

Replication of recombinant viruses encoding M2-A83 mutations at 32°C. To characterize the replication of recombinant influenza A viruses encoding M2-A83 mutations, a multiple-step growth curve was performed on MDCK and differentiated hNEC cultures. To mimic the natural infection temperature of the upper respiratory tract, the growth curves were performed at 32°C. All the recombinant viruses reached peak virus titers at 72 hpi, but viruses encoding M2-A83E had slightly faster kinetics compared to WT virus

at early times post infection in MDCK cells, though the differences were always less than 10-fold(Fig.12A). These data demonstrates that in MDCK cells under 32° C, M2-A83 mutation has little influence to virus replication.

In hNEC cultures, recombinant viruses encoding M2-A83V, A83E and A83K showed slightly increased kinetics of virus production but had identical peak virus titers when compared to WT virus(Fig.13A). Interestingly, the virus encoding M2-A83M was severely attenuated in its replication, showing up to 1000 fold less infectious virus production over multiple timepoints compared to WT virus. This hNEC growth curve demonstrated M2-A83 mutations have different effects on virus replication, depending on the amino acid substitution made.

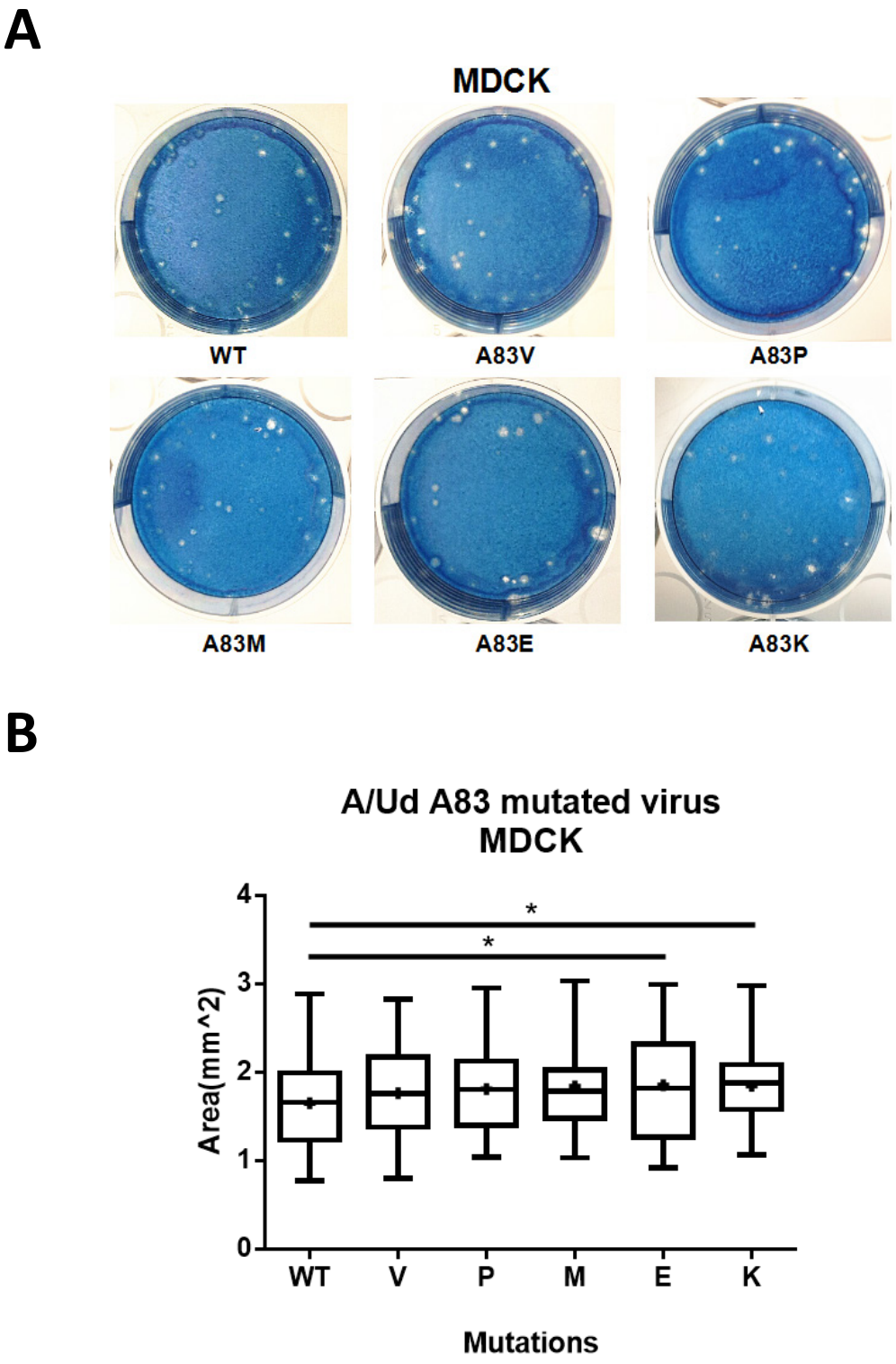
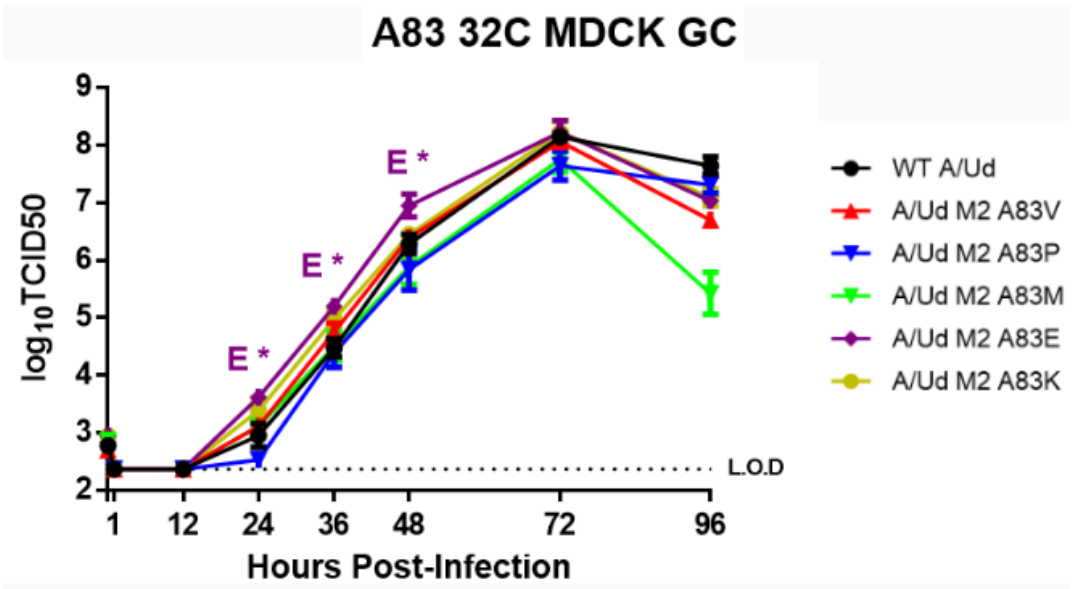


Figure. 11. Effect of amino acid substitutions at position 83 of the M2 protein on influenza A virus plaque morphology in MDCK cells.Plaque morphology of recombinant influenza A viruses encoding mutations at position 83 of the M2 protein.(B) Plaque area was calculated and mean(cross), median(line), 50% percentiles(box) and range(whiskers) are shown. Significant differences were determined by student t-test: * P≤0.05. At least 35 plaques were quantified for each virus.

A



B

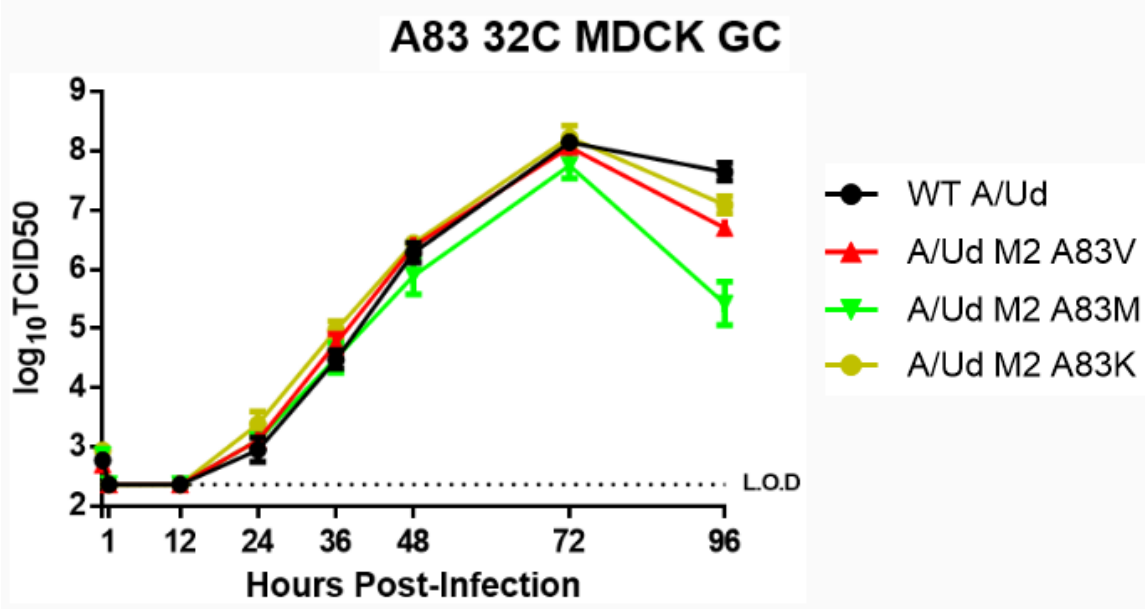
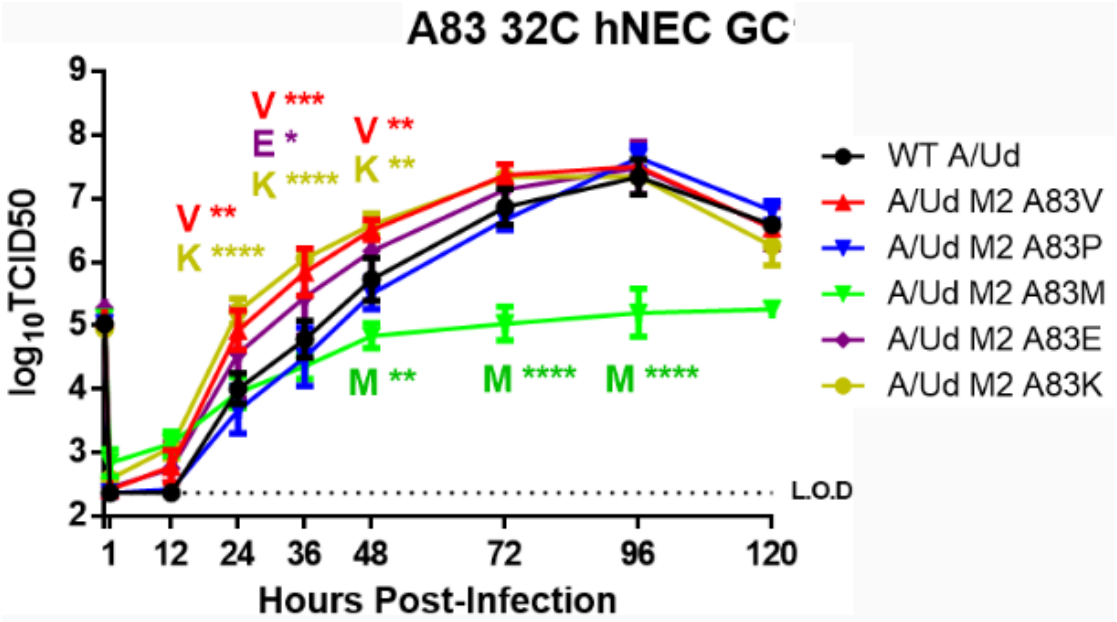


Figure. 12. Effect of amino acid substitutions at position 83 of the M2 protein on influenza A virus replication in MDCK cells at 32°C.(A) A multistep growth curve was performed on MDCK cells with the indicated recombinant viruses. In(B), representative viruses from(A) are shown. Statistical differences determined by MANOVA followed by Bonferroni post test: *=P<0.05; L.O.D=limit of detection=2.37.

A



B

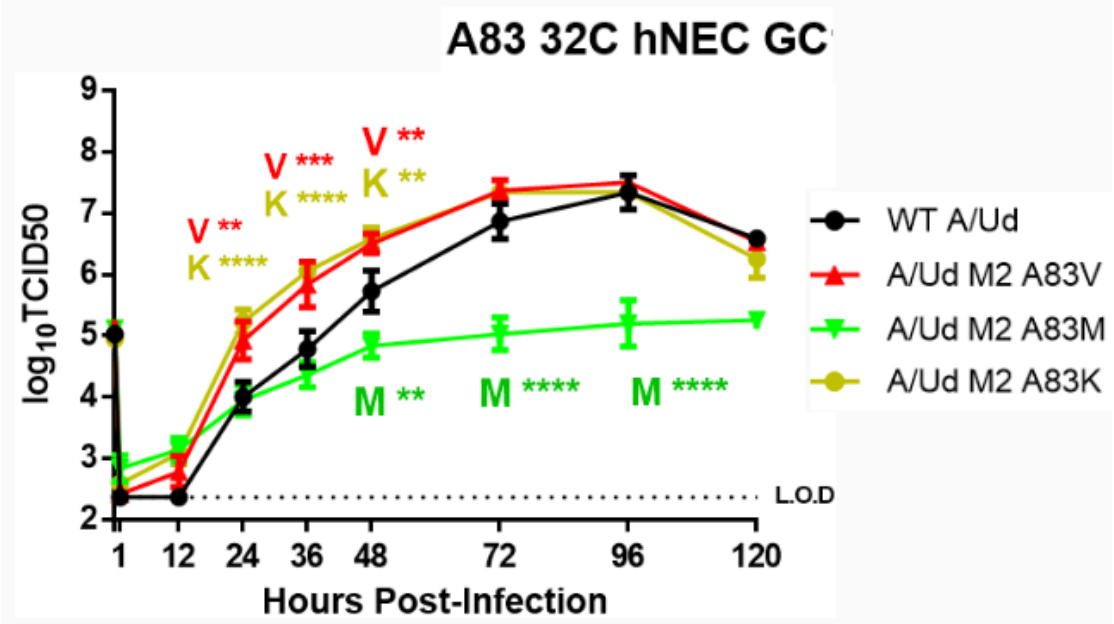


Figure. 13. Effect of amino acid substitutions at position 83 of the M2 protein on influenza A virus replication in hNEC cultures at 32°C(A) A multistep growth curve was performed on hNEC cultures with the indicated recombinant viruses. In(B), representative viruses from(A) are shown. Statistical differences determined by MANOVA followed by Bonferroni post test: *=P<0.05. **=P<0.01. ***=P<0.001. ****=P<0.0001. L.O.D=limit of detection=2.37

DISCUSSION

The cytoplasmic tail of the influenza A virus M2 protein contains 54 amino acids located at positions 44 to 97 of the protein(65). Besides the ion channel function which is important for virus entry, the M2 protein plays a role in the assembly of infectious virus particles(66). Various M2 protein truncations demonstrate the importance of amino acid sequences between positions 82 to 90. Disruption of these protein sequences led to a defect in virus replication. This thesis was focused on M2 amino acids 83 and 86, which were not mutated previously and could be important determinants of efficient virus replication(53). In our study, we attempted to examine the role of two specific amino acid position M2-A83 and M2-A86 in virus replication by characterizing their mutations in plaque assays and multiple-step growth curves. Our characterization experiments demonatrate that M2-A83 and A86 positions play roles in A/Udorn influenza virus replication. Mutations at these 2 positions can alter the replication kinetics and amount of infectious virus produced. Based on prior research on A/Udorn viruses with M2 protein truncations at position 70(M2stop70), M2 protein amino acid positions 70 to 90 are not important for M2 protein incorporation into progeny virus particles but M2stop70 viruses have reduced virus infectivity, decreased NP incorporation, and reduced viral RNA incorporation into progeny virus particles. This reduction can be restored by the expression of full-length M2

protein in *trans*(61). Although M2 cytoplasmic tail amino acid position 71 and 76 have been proved to be critical for virus replication(62), they may not be the only positions that are important because truncation at amino acid 82 also attenuates virus replication. Since both M2-A83 and M2-A86 position are located within the sequence 82-90, they may be part of a second region of the M2 cytoplasmic tail that is important in infectious virus production. To further investigate the role of mutations at positions 83 and 86 of the M2 protein, high MOI infections should be performed and the amount of viral NP and viral RNA packaged into progeny virus particles determined. I would predict that mutations that attenuate virus replication would reduce NP and viral RNA incorporation while mutations that improve virus replication should increase NP and viral RNA incorporation.

The structure of the M2 protein cytoplasmic tail from amino acids 72-97 has not been solved. According to the Chou and Fasman algorithm, amino acid positions 82 to 87 form an alpha-helix secondary protein structure(55,56). In analyzing our data on virus replication and the predicted secondary structure changes of different mutated amino acids, it is difficult to find any consistency between the two: In M2-A83, V and K promotes replication while M drastically inhibits progeny virus production. Although introducing V and K to M2-A83 position makes that 82-87 alpha-helix disrupted by 1-2 amino acids, introducing M does not make any structural difference. A similar situation occurs with the M2-A86 mutations. Based on the Chou and Fasman

prediction(55,56), S, E and K may cause similar structural changes in the 82-87 region, but my data shows only S and E but not K reduce the virus replication. These suggests that the alteration of virus replication may not be related to the Chou and Fasman predicted M2 cytoplasmic tail secondary structures, however, this awaits the determination of the actual M2 cytoplasmic tail structure.

The M2-A83 position has nearly 100% alanine for all human influenza A strains. M2-A86 position has alanine for most of human influenza A virus non-pandemic H1N1 strains(pH1N1), while M2-A86 has valine for the majority of pH1N1(54). Our data generated from growth curve suggests valine at A86 position has some inhibiting influence for virus replication of the H3N2 influenza A virus A/Udorn(Fig.9), but not as much as serine and glutamic acid. It will be interesting to investigate the role of position 86 in pH1N1 viruses and perhaps confirm that the M2-A86V substitution contributes to that viruses ability to replicate efficiently.

For both position 83 and 86, we generated panels of mutated virus based on introducing amino acids with different side chain characteristics. At position 86, LAIV strain mutation S and the basic amino acid E attenuate virus, which indicates that both hydrophobicity and polarity features of amino acid residues are factors responsible for the functions. This is also applicable to position 83, where the size of the hydrophobic side chain or the presence of the S-methyl thioether side chain cause mutation A83M to significantly

attenuate virus replication. To further study how the amino acid residue properties contribute to virus replication at each position, some additional mutations can be introduced into these positions. For example, an A86D(aspartic acid) mutation can be created and compared with A86E to determine the role of acidic side chains at this position.

Our mutations are generated based on A/Udorn/307/72(H3N2) virus backbone, which is a widely used laboratory research strain(67) and also used in the prior studies on the effect of truncating the M2 cytoplasmic tail(53). Therefore, our results are the outcome of mutations interacting with A/Udorn backbone genes. For the A86S mutation in particular, it will be especially important to study its effects in the LAIV genetic background to determine if that position contributes to LAIV attenuation. If this reverse mutation can weaken the attenuation phenotype, which means the containing virus would replicate better than normal LAIV strain, then position 86 can be determined to be an attenuation-related position of the M2 protein.

We used two types of cells in our studies: MDCK cells and hNEC cultures. Although MDCK cells are a widely used laboratory cell line to grow influenza virus and are easier and cheaper to use(68), they may not be the most relevant cell line for characterizing some aspects of influenza virus replication. Our results demonstrate the difference of using MDCK cells compared to hNEC cultures and - perhaps obviously - the more physiologically relevant hNECs are a better cell model to use for influenza research, especially for human

vaccine related experiments. MDCK cells are not able to show the variation of mutated strains we generated but hNECs can. This difference revealed by hNEC but not MDCK, is that specific mutations at position 86 resulted in viruses that replicated less effectively at 37° C than 32° C in hNEC, suggests that 86 position is related with a temperature sensitive phenotype that penetrates only on hNEC cultures(Fig.7A & Fig.9). Previous work focusing on the *ts* phenotype of cold adapted LAIV virus concluded that segments related to the *ts* phenotype are PB1, PB2 and NP, which carry 5 amino acid changes when LAIV is compared to its parental virus(63). However, their experiments are all based on MDCK cells. We discovered an additional *ts* phenotype from the M2-A86S mutations in hNECs, which suggests hNECs are a better cell culture model for influenza virus research. Of course, hNEC cultures are difficult to obtain and maintain so they are certainly not as convenient as MDCK cells. The choices of these two cells would be important for further influenza virus research.

Our research extends our understanding of influenza A virus replication and provides additional amino acid positions that can alter virus replication. This could be important information used for designing improved influenza LAIV vaccines. The current live-attenuated influenza vaccine are only approved for individuals between 2 to 49 years of age(69). However, these two groups of people indeed have higher needs of immunological protection than others due to their weaker immune responses(70). They urgently need a safer

live-attenuated vaccine for protection, and we may generate more attenuated influenza A virus strain to fit this need based on the points we found.

FUTURE DIRECTION

Our study identified specific mutations at two positions in the M2 protein that altered virus replication kinetics and infectious virus production. However, we still lack detailed knowledge about the core mechanisms responsible for these differences. Based on prior studies(61), we assume that this mechanism is that M2-A83 and M2-A86 positions are important for progeny virion genome packaging. To verify this assumption, it is necessary to measure the RNA amount of progeny virus of mutated viruses.

Also, as we demonstrate the attenuation of M2-A86S strain virus, characterizing similar mutations in the LAIV strain backbone would be informative. The contribution of M2 86 amino acids to LAIV attenuation need to be determined to see if the degree of attenuation is comparable to that seen with A/Udorn A86S virus.

We also poorly understand the impact of mutated virus infection to host immune response. Since we use hNECs to research, measuring the innate immune factors(interferon, chemokines and cytokines) produced post infection could be a reasonable point to start.

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Characterized generated viruses through plaque assays and multi-step growth curves in both MDCK and human nasal epithelial cells.

Analyzing characterization result for potential research directions.

2013/03-2014/07 **Researcher, Electric Potential fluctuation and peristltic behaviour of *P.polycephalum*, NJU**

Instructor: Prof. Changhong Liu, Prof. Jian Jing. Consultant: Prof. Andrew Adamatzky,

Studied on the growth characteristics of *Physarum polycephalum*

Analyzed obtained electric potential data.

2013/07-2013/09 **Research Assistant, Mutagenesis and Selection of Cellulase in *Humicola*. Chinese Academy of Agricultural Sciences(CAAS)**

Instructor: Prof. Guoqing Sun, Bo Liu, and Wei Zhang,

Introduced mutations altering cellulase activity in *Humicola* strains

Isolated high cellulase activity *Humicola* strains after mutation.

2012/02-2012/09 **Research Assistant, Determination of Contents of Soil Microorganism in Huaian, Jiangsu, NJU**

Instructor: Prof. Changhong Liu

Isolated microbes and measured concentrations of metal ions in soil samples.

Skills:

PCR
Mutagenesis
Transfection
Plaque assay and plaque picking
Multistep growth curve
TCID50 assay
DNA, RNA purification
Gel electrophoresis
Gel purification
Microscopy
R software, Graphpad Prism 6, Geneious
Microsoft Word, Excel, Powerpoint
Language: Chinese, English
Lab daily supply and maintainance management

Relevant Coursework:

Immunology; Fundamental and Advanced Virology; Parasitology with lab; Biochemistry with lab Molecular biology with lab; Organic Chemistry with lab; Microbiology with lab; Biostatistics with

lab
Statistics for laboratory Scientists; Practical Epidemiology for Basic Scientists

Other Experience:

Attendee of ASV2015: 34th Annual Meeting of the American Society of Virology, Ontario Canada
Volunteer at United Nations World Food Program(WFP), Beijing Liaison Office to assist China-WFP food fortification project
Internship at International Network for Bamboo and Rattan(INBAR), Headquarter in Beijing
Member and later Consultant of Student Union of Life Science School Human Resource Department, NJU

Reference Available upon request